PCT/GB2003/005192

INVESTOR IN PEOPLE

2 JAN 2005

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

26 JAN 2005

REC'D 21 AUG 2003
WIPO PCT

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

I also certify that the attached copy of the request for grant of a Patent (Form 1/77) bears a correction, effected by this office, following a request by the applicant and agreed to by the Comptroller-General.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed R. Mahoney

Dated 11 August 2003

BEST AVAILABLE COPY

Patents Form 1/77

Patents Act 1977
(Rule 16)

1/77

29JUL02 E736557-1 D00056
P01/7700 0.00-0217402.7

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office

Cardiff Road
Newport
South Wales
NP9 1RH

1. Your reference — P32590GB/NCB

2. Patent application number *(The Patent Office will fill in this part)* — 0217402.7

AE 4/77 28.10.02

26 JUL 2002

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

ROSLIN INSTITUTE (EDINBURGH)
Roslin
Midlothian
EH25 9PS
GB

CXR BIOSCIENCES LIMITED
DUNDEE TECHNOPOLE
JAMES LINDSAY PLACE
DUNDEE DD1 5JJ

Patents ADP number *(if you know it)* 8336422001

If the applicant is a corporate body, give the country/state of its incorporation — GB

4. Title of the invention

MULTI-REPORTER GENE MODEL FOR TOXICOLOGICAL SCREENING

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Kilburn & Strode
20 Red Lion Street
London
WC1R 4PJ

Patents ADP number *(if you know it)* 125001

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

YES

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	
Description	25
Claim *(s)*	4
Abstract	-
Drawing *(s)*	6 + 6

10. If you are also filing any of the following, state how many against each item.

Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Signature [signature] Date 26 July 2002

12. Name and daytime telephone number of person to contact in the United Kingdom

Mr Nick C Bassil
Tel: 020 7539 4200

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) If you need help to fill in this form or you have any questions, please contact the Patent Office on 0645 500505.

b) Write your answers in capital letters using black ink or you may type them.

c) If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.

d) If you have answered 'Yes' Patents Form 7/77 will need to be filed.

e) Once you have filled in the form you must remember to sign and date it.

f) For details of the fee and ways to pay please contact the Patent Office.

MULTI-REPORTER GENE MODEL FOR TOXICOLOGICAL SCREENING

The present invention relates to a non-invasive reporter gene system for the detection of gene activation events related to altered metabolic status in vivo or in vitro for use in toxicological screening.

Genes encode proteins. It is estimated that there at least 3 x 10^4 genes in the vertebrate genome but for a given cell only a subset of the total number of genes is active, with the subset differing between cells of different types and between different stages of development and differentiation (Cho & Campbell *Trends Genet.* **16** 409-415 (2000); Velculescu *et al Trends Genet.* **16** 423-425 (2000)). The DNA regulatory elements associated with each gene governs the decision as to which genes are active and which are not. Although comprising a number of defined elements these DNA sequences are collectively termed promoters (Tjian & Maniatis *Cell* **77** 5-8 (1994); Bonifer, *Trends Genet.* **16** 310-315 (2000); Martin, *Trends Genet* **17** 444-448 (2001)).

Gene activation occurs primarily at the transcriptional level. Transcriptional activity of a gene may be measured by a variety of approaches including RNA polymerase activity, mRNA abundance or protein production (Takano et al., 2002). These approaches are limited in that they require development of an assay suitable to each individual mRNA or protein product. To facilitate comparison of different promoters, rather than assaying individual gene products, reporter genes are often used (Sun *et al Gene Ther.* **8** 1572-1579 (2001); Franco *et al Eur. J. Morphol.* **39** 169-191 (2001); Hadjantonakis & Nagy, *Histochem. Cell. Biol.* **115** 49-58 (2001); Gorman *Mol. Cell. Biol.* **2** 1044-1051 (1982); Barash and Reichenstein, 2002; Zhang et al., 2001.).

The product (mRNA or protein) of a reporter gene allows an assessment of the transcriptional activity of a particular gene and can be used to distinguish cells, tissues or organisms in which the event has occurred from those in which it has not. On the whole reporter genes are foreign to the host cell or organism, allowing their activity to

be easily distinguished from the activity of endogenous genes. Alternatively the reporter may be marked or tagged so as to make it distinct from host genes.

Reporter genes are linked to the test promoter, enabling activity of the promoter gene to be determined by detecting the presence of the reporter gene product. Therefore, the main prerequisite for a reporter gene product is that it is easy to detect and quantify. In some cases, but not all, the reporter gene has enzymatic activity that catalyses the conversion of a substrate into a measurable product.

A classical example is the bacterial chloramphenicol acetyl transferase (CAT) gene. CAT activity can be measured in cell extracts as conversion of added non-acetylated chloramphenicol to the acetylated form of chloramphenicol by chromatography (Gorman *Mol. Cell. Biol.* **2** 1044-1051 (1982)). Similar strategies enable the use of the firefly luciferase gene as a reporter. In this instance it is the light produced by bioluminescence of the luciferin substrate that is measured.

Some reporters also benefit from the visual detection assays that allow *in situ* analysis of reporter activity. A frequently used example would be β-galactosidase (Lac Z), where the addition of an artificial substrate, X-gal, enables reporter activity to be detected by the appearance of blue colouration in the sample. As it is accumulative it effectively provides an historical record of its induction. This is particularly useful for measuring transient responses where a promoter is activated for only a short time before being rapidly inactivated. This reporter has been successfully used both in cultured cells and in vivo (Campbell *et al J. Cell. Biol.* **109** 2619-2625 (1996)), though its suitability for in vivo use has been questioned in some reports (Sanchez-Ramnos *et al Cell Transplant.* **9** 657-667 (2000); Montoliu *et al Transgenic Res.* **9** 237-239 (2000); Cohen-Tannoudji *et al Transgenic Res.* **9** 233-235 (2000)). It has been demonstrated that Lac Z in combination with fluorescent substrates can enable the sorting of cells that express the reporter by use of a fluorescence-activated cell sorter (FACS) (Fiering *et al Cytometry* **12** 291-301 (1991)).

In other systems, the reporter product itself is directly detected, removing the need for a substrate. Green fluorescent protein has become on of the most commonly used examples of this category of reporter (Ikawa *et al Curr. Top. Dev. Biol.* **44** 1-20 (1997)). This autofluorescing protein was derived from the bioluminescent jellyfish *Aequoria victoria*. Several colour spectral variants of this reporter have been developed (Hadjantonakis & Nagy, *Histochem. Cell. Biol.* **115** 49-58 (2001)).

Recently reporter systems based on energy emission systems have been developed. These include single photon emission computed tomography (SPECT) and positron emission tomography (PET) though these require the introduction of a radiolabelled isotope probe in to the host cell or animal that is then modified by the target reporter gene. For example the PET system measures reporter sequestering of the positron emitting probe (Sun *et al Gene Ther.* **8** 1572-1579 (2001)). These are summarised as follows:

Established reporter	
Enzymatic	**Light based**
alkaline phosphatase	Green fluorescent protein
Beta galactosidase	dsRed
Thymidine kinase	Luciferase
Neomycin resistance	
Chloramphenicol acetyl transferase	
Growth hormone	

Many tried and tested reporter systems have been developed but nevertheless share certain limitations. Those based on prokaryote genes often suffer poor expression in transgenic mammals (Montoliu *et al Transgenic Res.* **9** 237-238 (2000); Cohen-Tannoudji *et al Transgenic Res.* **9** 233-235 (2000)). Furthermore the presence of prokaryote DNA sequences has been implicated in the suppression of expression from adjacent eukaryote transgenes as have the presence of intronless, cDNA based eukaryote gene sequences (Clark et al., 1997).

Most of the current reporters, whilst useful for monitoring expression under certain circumstances, have certain limitations. Many accumulate in cells and are not useful for monitoring changes in promoter activation over time. Perhaps more importantly detection of expression necessitates the fixing of cultured cells or the sacrifice of transgenic animals, thus limiting reporters to invasive detection strategies. There are a few exceptions; These include the use of growth hormone (Bchini *et al Endocrinology* **128** 539-546 (1991)). However its high biological activity effectively limit its widespread applicability. Another enzyme that has been used in vivo is a secreted version of alkaline phosphatase (SEAP) (Nilsson *et al Cancer Chemother. Pharmacol.* **49** 93-100 (2002); Durocher *Nucl. Acids. Res.* **30** E9 (2002)) though again, the potential biological effects resulting from its heterologous expression remain untested. GFP has been detected in whole animals and though possessing relatively low biological activity its use has so far been limited to neonatal and nude mice in which both internal tissue and dermal fluorescence are more readily observed. In addition there has been a report that GFP is cytotoxic (Liu *et al Biochem. Biophys. Res. Comm.* **260** 712-717 (1999)). Although reporter systems based on tomography allow monitoring of reporter expression in internal tissues they require addition of exogenously added substrates that could potentially confound results by influencing expression of the reporter. Additionally they can lack the sensitivity required for quantitative analysis of reporter expression.

There is therefore a need for a reporter system that overcomes some or all of these limitations. Primarily it should be non-invasive inasmuch as its detection does not involve addition of an external substrate or sacrifice of transgenic animals. This would also ideally stipulate that the reporter be secreted (in vitro and in vivo) or excreted (in vivo). Secondly it should be biologically neutral with regard to the test expression system so that no phenotypic effects either confound readout from the system or affect the health of the transgenic animal. Thirdly a family of reporters sharing similar and therefore predictable characteristics allowing comparison between reporters is required. This may be achieved if members share a common structure or backbone.

A system satisfying these requirements has now been found. The members of the lipocalin protein family fulfil the necessary characteristics for a non-invasive reporter.

According to a first aspect of the invention, there is provided a nucleic acid construct comprising (i) a nucleic acid sequence encoding a member of the lipocalin protein family, and (ii) and nucleic acid sequence encoding a peptide sequence of from 5 to 250 amino acid residues

The lipocalins are a diverse family of small molecule transporter proteins that share a common conserved gene structure (Flower *et al Biochim. Biophys Acta* **1482** 9-24 (2000)). Members of this family are small in size with the majority falling into the 18-25kD range. Some are naturally secreted, e.g. ovine betalactoglobulin (BLG) (accession No. X12817), or excreted e.g. murine major urinary protein (MUP) (e.g. accession No. NM 031188) and rat α-2-urinary globulin (α-2u) (accession number M27434). Lipocalin reporters will preferably be either MUP, BLG or α-2u but could be chosen from the following list of other lipocalin family members shown in Table 1:

Table 1

Protein	Subunit molecular mass	pI	No. residues	Oligomeric State	Glycosyln.	No. S=S	Abbr. / ref
Kernel lipocalins							
Retinol-binding protein	21.0	5.5	183	Monomer	-	3	RBP (1), (2)
Purpurin	20.0		175				PURP (3)
Retinoic acid-binding protein	18.5	5.2	166	Monomer	-	1	RABP (4)
α_{2u}-Globulin	18.7	5.7–6.7	162	Dimer	-	1	A2U (5)-(7)
Major urinary protein	17.8	5.5–5.7	161	Dimer	-	1	MUP (8)–(10)
Bilin-binding protein	19.6		173	Tetramer	-	2	BBP (11)
α-Crustacyanin	350.0	4.3–4.7	174/181	Octamer of heterodimers	-	2/2	(12) (13)
Pregnancy protein 14	56.0		162	Homodimer	+		PP14 (15)
β-Lactoglobulin	18.0	5.2	162	Dimer/ monomer	-	2	Blg (16)–(18)

Protein	Subunit molecular mass	pI	No. residues	Oligomeric State	Glycosyln.	No. S=S	Abbr. / ref
α_1-Microglobulin	31.0	4.3–4.8	188	Monomer +complexes	+	1	A1M (19)
C8γ	22.0		182	Part of complex	-	1	C8γ (20)
Apolipoprotein D	29.0–32.0	4.7–5.2	169	Dimer +complexes	+	2	ApoD (21)-(23)
Lazarillo	45.0		183	Monomer	+	+	LAZ (24)
Prostaglandin D synthase	27.0	4.6	168	Monomer	+	1	PGDS (25)
Quiescence-specific protein	21.0	6.3	158			1	QSP (26)-(28)
Neutrophil lipocalin	25.0		179	Monomer/ Dimer +complexes			NGAL (29)-(32)
Choroid plexus protein	20.0		183	Monomer	-		(33)
Outlier lipocalins							
Odorant-binding protein	37.0–40.0	4.7	159	Dimer		0	OBP (34)-(36)
von Ebner's-gland protein	18.0	4.8–5.2	170	Dimer		1	VEGP (37)-(40)
α_1-Acid glycoprotein	40.0	3.2	183	Monomer	+	2	AGP (41)(42)
Probasin	20.0	11.5	160				PBAS (43)
Aphrodisin	17.0		151		+	2	(44)

"Glycosyln". = glycosylation

"No. S=S" = no. of disulphides

References:

(1) Cogan *et al Eur J. Biochem* **65**, 71-78 (1976).

(2) Hase *et al J. Biochem* **79** 373-380 (1976)

(3) Berman *et al Cell* **51** 135-142 (1987)

(4) Newcomer, M. E. & Ong, D. E., *J. Biol. Chem.* **265** 12876-12879 (1990)

(5) Borghoff *et al Ann. Rev. Pharmacol. Toxicol.* **30** 349-367 (1990)

(6) Cavaggioni *et al Comp. Biochem. Physiol.* **96B** 513-520 (1990)

(7) Borghoff *et al Toxicol. Appl. Pharmacol.* **107** 228-238 (1991)

(8) Shaw *et al Cell* **32** 755-761 (1983)

(9) Cavaggioni *et al Comp. Biochem. Physiol.* **96B** 513-520 (1990)

(10) Borghoff *et al Toxicol. Appl. Pharmacol.* **107** 228-238 (1991)

(11) Riley *et al J. Biol. Chem.* **259** 13159-13165 (1984)

(12) Britton *et al* in *"Carotenoid Chemistry and Biochemistry"*, eds. Britton, G. & Goodwin, T. W., 237-253, Pergamon Press, Oxford (1982)

(13) Zagalsky *et al Comp. Biochem. Physiol.* **97B** 1-18 (1990)

(14) Morrow *et al Am. J. Pathol.* **145** 1485-1495 (1995)

(15) Hambling *et al* in *"Advanced Dairy Chemistry"*, volume 1, ed. Fox, P. F.,, 140-190, Elsevier Applied Science London (1992)

(16) Dodin *et al Eur. J. Biochem.* **193** 697-700 (1990)

(17) Dufour *et al FEBS Lett* **277** 223-226 (1991)

(18) Escribano *et al Biochem. Biophys. Res. Comm.* **155** 1424-1429 (1988)

(19) Haefliger *et al Mol. Immunol.* **28** 123-131 (1991)

(20) Balbin *et al Biochem. J.* **271** 803-807 (1990)

(21) Peitsch, M. C. & Boguski, M. S., *New Biol.* **2** 197-206 (1990)

(22) Morais Cabral *et al FEBS Lett* **366** 53-56 (1995)

(23) Ganfornia *et al Development* **121** 123-134 (1995)

(24) Urade *et al J. Biol. Chem.* **264** 1041-1045 (1988)

(25) Cancedda *et al J. Cell Biol.* **107** 2455-2463 (1990)

(26) Cancedda *et al Biochem. Biophys. Res. Comm.* **168** 933-938 (1990)

(27) Nakano, T. & Graf, T. *Oncogene* **7** 527-534 (1992)

(28) Hraba-Renevey *et al Oncogene* **4** 601-608 (1989)

(29) Meheus *et al J. Immunol.* **151** 1535-1547 (1993)

(30) Liu, Q., & Nilsen-Hamilton, M. *J. Biol. Chem.* **270** 22565-22570 (1995)

(31) Kasik, J. W. & Rice, E. J. *Am. J. Obstet. Gynecol.* **173** 613-617 (1995)

(32) Achen *et al J. Biol. Chem.* **267** 23170-23174 (1992)

(33) Snyder *et al J. Biol. Chem.* **263** 13971-13974 (1988)

(34) Lee *et al Science* **235** 1053-1056 (1987)

(35) Cavaggioni *et al FEBS Lett* **212** 225-228 (1987)

(36) Kock *et al Physiol. Behav.* **56** 1173-1177 (1994)

(37) Schmale *et al Ciba Found. Symp.* **179** 167-185 (1993)

(38) Redl *et al J. Biol. Chem.* **267** 20282-20827 (1992)

(39) Glasgow *et al Curr. Eye Res.* **14** 363-372 (1995)

(40) Kremer *et al Pharmacol. Rev.* **40** 1-40 (1988)

(41) Arnaud *et al Methods Enzymol.* **163** 418-431 (1988)

(42) Matuo *et al Biochem. Biophys. Res. Comm.* **118** 467-473 (1984)

(43) Henzel *et al J. Biol. Chem.* **263** 16682-16687 (1988)

(44) Magert *et al Proc. Nat'l Acad. Sci. USA* **92** 2091-2095 (1995)

With the addition of peptide tags to a chosen lipocalin reporter there is provided a useful sub-family of reporter proteins. Essentially it allows generation of a large number of reporters from a single lipocalin where that lipocalin acts as the carrier for a range of peptides that can be clearly differentiated from one another by a range or biological or physical assay techniques. For example it has been demonstrated that a casein kinase recognition sequence engineered in exon 3 of the ovine betalactoglobulin (BLG) gene resulted in expression of a novel form of BLG containing an active kinase substrate in one of the surface loops of the protein in transgenic mice (McClenaghan *et al Protein Eng.* **12** 259-264 (1999)).

The position of the peptide tag may be at the amino terminal or carboxy terminal or inserted internally with respect to the amino acid sequence of the reporter. All three examples are represented in Figure 1.

The peptide tag can be a sequence consisting of between 5 to 250 amino acids. Suitably, in the ranges of from, 5 to 50, 10 to 60, 20 to 70, 30 to 80, 40 to 90, and so on. In some embodiments of the invention peptides may be required to consist of a greater number of amino acids than 250 residues.

In a preferred embodiment of the invention the peptide tag may be an epitope, that is a defined amino acid sequence from a protein with a fully characterised cognate antibody. The skilled person can select such epitopes based on sequences identified as possessing antigenic properties. In certain embodiments of the invention the epitope tag may be the amino acid sequence below from the c-myc oncogene (Evans *et al Mol. Cell. Biol.* **5** 3610-3616 (1985)):

-Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-

or it may be the amino acid sequence from the simian virus V5 protein (Southern *et al* *J. Gen. Virol.* **72** 1551-1557 (1991)), shown below:

-Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr-

In certain embodiments of the invention, the epitope may be selected from but not limited to the c-myc and V5 proteins.

Preferably the epitope tag is recognised by its cognate antibody irrespective of whether it is located at the amino terminal, carboxy terminal or in an internal domain of the reporter protein.

In another embodiment of the invention the peptide tag may possess enzymatic activity that converts a substrate to a form that is readily detectable by an assay. For example a kinase activity specifying phosphorylation of another protein or peptide substrate that could be added to the secreted or excreted analyte along with a phosphate group donor. Detection could be achieved using an immunological assay based on detection by an antibody specifically recognising the phosphorylated version of the tagged reporter protein. Alternatively the use of phosphate radiolabelled with an isotope of phosphorous such as ^{32}P or ^{33}P. Other enzymic modifications include for example acetylation, sulphation and glycosylation.

Other embodiments of this aspect could include, for example site of interaction with protein other than antibody e.g. lectin binding site, or modification of tag by e.g. addition of amino acid multimer such as polylysine; or incorporation of a fluorochrome.

According to the various embodiments of this aspect of the invention, the promoter will preferably be of mammalian origin, but also may be from a non-mammalian

animal, plant, yeast or bacteria. The promoter may be selected from but is not limited to promoter elements of the following inducible genes:

whose expression is modified in response to disturbances in the homeostatic state of DNA in the cell. These disturbances may include chemical alteration of nucleic acids or precursor nucleotides, inhibition of DNA synthesis and inhibition of DNA replication. The sequence can be selected from but not limited to the group consisting of c-myc (Hoffman *et al Oncogene* **21** 3414-3421), p21/WAF-1 (El-Diery *Curr. Top. Microbiol. Immunol.* **227** 121-137 (1998); El-Diery *Cell Death Differ.* **8** 1066-1075 (2001); Dotto *Biochim. Biophys. Acta* **1471** 43-56 (2000)), MDM2 (Alarcon-Vargas & Ronai *Carcinogenesis* **23** 541-547 (2002); Deb & Front *Bioscience* **7** 235-243 (2002)), Gadd45 (Sheikh *et al Biochem. Pharmacol.* **59** 43-45 (2000)), FasL (Wajant *Science* **296** 1635-1636 (2002)), GAHSP40 (Hamajima *et al J. Cell. Biol.* **84** 401-407 (2002)), TRAIL-R2/DR5 (Wu *et al Adv.Exp. Med. Biol.* **465** 143-151 (2000); El-Diery *Cell Death Differ.* **8** 1066-1075 (2001)), BTG2/PC3 (Tirone *et al J. Cell. Physiol.* **187** 155-165 (2001));

whose transcription is modified in response to oxidative stress. The sequence can be selected from but not limited to the group consisting of MnSOD and/or CuZnSOD (Halliwell *Free Radic. Res.* **31** 261-272 (1999); Gutteridge & Halliwell *Ann. NY Acad. Sci.* **899** 136-147 (2000)), IκB (Ghosh & Karin *Cell* **109** Suppl.., S81-96 (2002)), ATF4 (Hai & Hartman *Gene* **273** 1-11 (2001)), xanthine oxidase (Pristos *Chem. Biol. Interact.* **129** 195-208 (2000)), COX2 (Hinz & Brune *J. Pharmacol. Exp. Ther.* **300** 376-375 (2002)), iNOS (Alderton *et al Biochem. J.* **357** 593-615 (2001)), Ets-2 (Bartel *et al Oncogene* **19** 6443-6454 (2000)), FasL/CD95L (Wajant *Science* **296** 1635-1636 (2002)), γGCS (Lu *Curr. Top. Cell. Regul.* **36** 95-116 (2000); Soltaninassab *et al J. Cell. Physiol.* **182** 163-170 (2000)), ORP150 (Ozawa *et al Cancer Res.* **61** 4206-4213 (2001); Ozawa *et al J. Biol. Chem.* **274** 6397-6404 (1999)).

whose expression is modified in response to hepatotoxic stress. The sequence can be selected from but not limited to the group consisting of Lrg-21 (Drysdale *et al Mol. Immunol.* **33** 989-998 (1996)), SOCS-2 and/or SOCS-3 (Tollet-Egnell *et al Endocrinol.* **140** 3693-3704 (1999), PAI-1 (Fink *et al Cell. Physiol. Biochem.* **11** 105-114 (2001)), GBP28/adiponectin (Yoda-Murakami *et al Biochem. Biophys. Res. Commun.* **285** 372-377 (2001)), α-1 acid glycoprotein (Komori *et al Biochem Pharmacol.* **62** 1391-1397 (2001)), metallothioneine I (Palmiter *et al Mol. Cell. Biol.* **13** 5266-5275 (1993)), metallothioneine II (Schlager & Hart *App. Toxicol.* **20** 395-405 (2000)), ATF3 (Hai & Hartman *Gene* **273** 1-11 (2001)), IGFbp-3 (Popovici *et al J. Clin. Endocrinol. Metab.* **86** 2653-2639 (2001)), VDGF (Ido *et al Cancer Res.* **61** 3016-3021 (2001)) and HIF1α (Tacchini *et al Biochem. Pharmacol.* **63** 139-148 (2002)).

whose expression is modified in response to a pro-apoptotic stimulus. The sequence can be selected from but not limited to the group consisting of Gadd 34 (Hollander *et al J. Biol. Chem.* **272** 13731-13737 (1997)), GAHSP40 (Hamajima *et al J. Cell. Biol.* **84** 401-407 (2002)), TRAIL-R2/DR5 (Wu *et al Adv.Exp. Med. Biol.* **465** 143-151 (2000); El-Diery *Cell Death Differ.* **8** 1066-1075 (2001)), c-fos (Teng *Int. Rev. Cytol.* **197** 137-202 (2000)), CHOP/Gadd153 (Talukder *et al Oncogene* **21** 4280-4300 (2002)), APAF-1 (Cecconi & Gruss *Cell. Mol. Life Sci.* **5** 1688-1698 (2001)), Gadd45 (Sheikh *et al Biochem. Pharmacol.* **59** 43-45 (2000(), BTG2/PC3 (Tirone *J. Cell. Physiol.* **187** 155-165 (2001)), Peg3/Pwl (Relaix *et al Proc. Nat'l Acad. Sci. USA* **97** 2105-2110 (2000)), Siah 1a (Maeda *et al FEBS Lett.* **512** 223-226 (2002)), S29 ribosomal protein (Khanna *et al Biochem. Biophys. Res. Commun.* **277** 476-486 (2000)), FasL/CD95L (Wajant *Science* **296** 1635-1636 (2002)), tissue tranglutaminase (Chen & Mehta *Int. J. Cell. Biol.* **31** 817-836 (1999)), GRP78 (Rao *et al FEBS Lett.* **514** 122-128 (2002)), Nur77/NGFI-B (Winoto *Int. Arch. Allergy Immunol.* **105** 344-346 (1994)), CyclophilinD (Andreeva *et al Int. J.*

Exp. Pathol. **80** 305-315 (1999)), p73 (Yang *et al Trends Genet.* **18** 90-95 (2002)) and Bak (Lutz *Biochem. Soc. Trans.* **28** 51-56 (2000)).

whose expression is modified in response to the administration of chemicals or drugs. The sequence can be selected from but not limited to the list comprised of xenobiotic metabolising cytochrome p450 enzymes from the 2A, 2B, 2C, 2D, 2E, 2S, 3A, 4A and 4B gene families (Smith *et al Xenobiotica* **28** 1129-1165 (1998); Honkaski & Negishi *J. Biochem. Mol. Toxicol.* **12** 3-9 (1998); Raucy *et al J. Pharmacol. Exp. Ther.* **302** 475-482 (2002); Quattrochi & Guzelian *Drug Metab. Dispos.* **29** 615-622 (2001)).

The promoter element may also be a synthetic promoter sequence comprised of a minimal eukaryote consensus promoter operatively linked to one or more sequence elements known to confer transcriptional inducibility in response to specific stimulus. A minimal eukaryotic consensus promoter is one that will direct transcription by eukaryotic polymerases only if associated with functional promoter elements or transcription factor binding sites. An example of which is the PhCMV*-1 (Furth *et al Proc. Nat'l Acad. Sci. USA* **91** 9302-9306 (1994)). Sequence elements known to confer transcriptional induction in response to specific stimulus include promoter elements (Montoliu *et al Proc. Nat'l Acad. Sci. USA* **92** 4244-4248 (1995)) or transcription factor binding sites; these will be chosen from but are not limited to the list comprising the aryl hydrocarbon (Ah)/Ah nuclear translocator (ARNT) receptor response element, the antioxidant response element (ARE), the xenobiotic response element (XRE).

According to a second aspect of the invention there is provided a nucleic acid construct comprising a stress inducible promoter operatively isolated from a nucleic acid sequence encoding a member of the lipocalin protein family by a nucleotide sequence flanked by nucleic acid sequences recognised by a site specific recombinase, or by insertion such that it is inverted with respect to the transcription unit encoding a member of the lipocalin protein family. The recombinase recognition sites are

arranged in such a way that the isolator sequence is deleted or the inverted promoter's orientation is reversed in the presence of the recombinase. The construct also comprises a nucleic acid sequence comprising a tissue specific promoter operatively linked to a gene encoding the coding sequence for the site specific recombinase.

This aspect allows for detecting reporter transgene induction in specified tissues only. By controlling the appropriate recombinase expression using a tissue specific promoter, the inducible transgene will only be viable in those tissues in which the promoter is active. For example, by driving recombinase activity from a liver specific promoter, only the liver will contain re-arranged reporter construct, and hence will the only tissue in which reporter induction can occur.

The recombination event producing an active reporter transcription unit may therefore only take place in tissues where the recombinase is expressed. In this way the reporter may only be expressed in specified tissue types where expression of the recombinase results in a functional transcription unit comprised of the inducible promoter linked to the promoter. Site specific recombinase systems know to perform such a function include the bacteriophage P1 cre-lox and the bacterial FLIP systems. The site specific recombinase sequences may therefore be two *lox*P sites of bacteriophage P1

The use of site specific recombination systems to generate precisely defined deletions in cultured mammalian cells has been demonstrated. Gu *et al.* (*Cell* **73** 1155-1164 (1993)) describe how a deletion in the immunoglobulin switch region in mouse ES cells was generated between two copies of the bacteriophage P1 *lox*P site by transient expression of the Cre site-specific recombinase, leaving a single *lox*P site. Similarly, yeast FLP recombinase has been used to precisely delete a selectable marker defined by recombinase target sites in mouse erythroleukemia cells (Fiering *et al.*, *Proc. Nat'l. Acad. Sci. USA* **90** 8469-8473 (1993)). The Cre *lox* system is exemplified below, but other site-specific recombinase systems could be used.

A construct used in the Cre *lox* system will usually have the following three functional elements:

1. The expression cassette;

2. A negative selectable marker (e.g. Herpes simplex virus thymidine kinase (TK) gene) expressed under the control of a ubiquitously expressed promoter (e.g. phosphoglycerate kinase (Soriano *et al.*, *Cell* **64** 693-702 (1991)); and

3. Two copies of the bacteriophage P1 site specific recombination site *lox*P (Baubonis *et al.*, *Nuc. Acids. Res.* **21** 2025-2029 (1993)) located at either end of the DNA fragment.

This construct can be eliminated from host cells or cell lines containing it by means of site specific recombination between the two *lox*P sites mediated by Cre recombinase protein which can be introduced into the cells by lipofection (Baubonis *et al.*, *Nuc. Acids Res.* **21** 2025-2029 (1993)). Cells which have deleted DNA between the two *lox*P sites are selected for loss of the TK gene (or other negative selectable marker) by growth in medium containing the appropriate drug (ganciclovir in the case of TK).

According to the third aspect of the invention there is provided a host cell transfected with a nucleic acid construct according to any one of the previous aspects of the invention. The cell type is preferably of human or non-human mammalian origin but may also be of other animal, plant, yeast or bacterial origin.

According to the fourth aspect of the invention, there is provided a transgenic non-human animal in which the cells of the non-human animal express the protein encoded by the nucleic acid construct according to any one of the previous aspects of the invention. The transgenic animal is preferably a mouse but may be another mammalian species, for example another rodent, e.g. a rat or a guinea pig, or another species such as rabbit, or a canine or feline, or an ungulate species such as ovine,

porcine, equine, caprine, bovine, or a non-mammalian animal species, e.g. an avian (such as poultry, e.g. chicken or turkey)..

In embodiments of the invention relating to the preparation of a transfected host cell or a transgenic non-human animal comprising the use of a nucleic acid construct as previously described, the cell or non-human animal may be subjected to further transgenesis, in which the transgenesis is the introduction of an additional gene or genes or protein-encoding nucleic acid sequence or sequences. The transgenesis may be transient or stable transfection of a cell or a cell line, an episomal expression system in a cell or a cell line, or preparation of a transgenic non-human animal by pronuclear microinjection, through recombination events in embryonic stem (ES) cells or by transfection of a cell whose nucleus is to be used as a donor nucleus in a nuclear transfer cloning procedure.

Methods of preparing a transgenic cell or cell line, or a transgenic non human animal, in which the method comprises transient or stable transfection of a cell or a cell line, expression of an episomal expression system in a cell or cell line, or pronuclear microinjection, recombination events in ES cells, or other cell line or by transfection of a cell line which may be differentiated down different developmental pathways and whose nucleus is to be used as the donor for nuclear transfer; wherein expression of an additional nucleic acid sequence or construct is used to screen for transfection or transgenesis in accordance with the first, second, third, or fourth aspects of the invention. Examples include use of selectable markers conferring resistance to antibiotics added to the growth medium of cells. E.g. neomycin resistance marker conferring resistance to G418. Further examples involve detection using nucleic acid sequences that are of complementary sequence and which will hybridise with, or a component of, the nucleic acid sequence in accordance with the first, second, third, or fourth aspects of the invention. Examples would include Southern blot analysis, northern blot analysis and PCR.

According to the fifth aspect of the invention, there is provided the use of a nucleic acid construct in accordance with any one of the first, second, third, or fourth aspects of the invention for the detection of a gene activation event resulting from a change in altered metabolic status in a cell *in vitro* or *in vivo*.

The gene activation event may be the result of induction of toxicological stress, metabolic changes, disease that may or may not be the result of viral, bacterial, fungal or parasitic infection.

According to the sixth aspect of the invention there is provided the use of a nucleic acid construct comprising a nucleic acid sequence encoding a member of the lipocalin protein family, wherein said lipocalin protein is heterologous to the cell in which it is expressed, for the detection of a gene activation event resulting from a change in altered metabolic status in a cell *in vitro* or *in vivo*.

The gene activation event may be the result of induction of toxicological stress, metabolic changes, disease that may or may not be the result of viral, bacterial, fungal or parasitic infection.

Uses in accordance with the fifth and sixth aspects of the invention also extend to the detection of disease states or characterisation of disease models in a cell, cell line or non human transgenic animal where a change in the gene expression profile within a target cell or tissue type is altered as a consequence of the disease. Diseases in the context of this aspect of the invention which are detectable under the methods disclosed may be defined as infectious disease, cancer, inflammatory disease, cardiovascular disease, metabolic disease, neurological disease and disease with a genetic basis.

An additional use in accordance with this aspect of the invention involves the growth of a transfected cell line in accordance with the third aspect in a suitable immunocompromised mouse strain (referred to as a xenograft), for example, the nude

mouse, wherein an alteration in the expression of the reporter described in the first or second aspects of the invention may be used as a measure of altered metabolic status of the host as a result of toxicological stress, metabolic changes, disease with a genetic basis or disease that may or may not be the result of viral, bacterial, fungal or parasitic infection. The scope of this use may also be of use in monitoring the effects of exogenous chemicals or drugs on the expression of the reporter construct.

The fifth and sixth aspects of the invention extend to methods of detecting a gene activation event in vitro or in vivo.

In an embodiment according to the fifth aspect of the invention, the method comprises assaying a host cell stably transfected with a nucleic acid construct in accordance with any one of the first or second aspects of the invention, or a transgenic non-human animal according to the fourth aspect of the invention, in which the cell or animal is subjected to a gene activation event that is signalled by expression of a peptide tagged lipocalin reporter gene.

In an embodiment according to the sixth aspect of the invention, the method comprises assaying a host cell stably transfected with a nucleic acid construct comprising a nucleic acid sequence encoding a member of the lipocalin protein family, wherein said lipocalin protein is heterologous to the cell in which it is expressed, or a transgenic non-human animal whose cells express such a construct, in which the cell or animal is subjected to a gene activation event that is signalled by expression of a peptide tagged lipocalin reporter gene.

Accordingly there is provided a method of screening for, or monitoring of toxicologically induced stress in a cell or a cell line or a non-human animal, comprising the use of a cell, cell line or non human animal which has been transfected with or carries a nucleic acid construct as described above.

Toxicological stress may be defined as DNA damage, oxidative stress, post translational chemical modification of cellular proteins, chemical modification of cellular nucleic acids, apoptosis, cell cycle arrest, hyperplasia, immunological changes, effects consequent to changes in hormone levels or chemical modification of hormones, or other factors which could lead to cell damage.

Accordingly, there is also provided a method for screening and characterising viral, bacterial, fungal, and parasitic infection comprising the use of a cell, cell line or non human animal which has been transfected with or carries a nucleic acid construct as described above.

Accordingly, there is additionally provided a method for screening for cancer, inflammatory disease, cardiovascular disease, metabolic disease, neurological disease and disease with a genetic basis comprising the use of a cell, cell line or non human animal which has been transfected with or carries a nucleic acid construct as described above.

In these contexts the cell may be transiently transfected, maintaining the nucleic acid construct as described above episomally and temporarily. Alternatively cells are stably transfected whereby the nucleic acid construct is permanently and stably integrated into the transfected cells' chromosomal DNA.

Also in this context transgenic animal is defined as a non human transgenic animal with the nucleic acid construct as defined above preferably integrated into its genomic DNA in all or some of its cells.

Expression of the peptide tagged lipocalin protein in respect of the fifth aspect of the invention can be assayed for by measuring levels of the lipocalin protein in cell culture medium or purified or partially purified fractions thereof.

Lipocalins are known to be secreted into body fluids and some are known to be eliminated in urine. Expression of the peptide tagged lipocalin protein in accordance with the fourth aspect of the invention therefore can be assayed for by measuring levels of lipocalin secreted into harvestable body fluids. In a preferred embodiment of the invention the body fluid will be urine, but may also be selected from the list including milk, saliva, tears, semen, blood and cerebrospinal fluid, or purified or partially purified fractions thereof.

Detection and quantification of the tagged lipocalins secreted from cultured cells into tissue culture medium or transgenic non-human animal body fluid may be achieved using a number of methods known to those skilled in the art:

1. Immunological methods.

(i) The assay may be an ELISA whereby an antibody or antiserum containing a single or mixture of antibodies recognising either the lipocalin reporter itself or the peptide tag attached to and is used as a capture antibody to coat a microtitre plate or other medium suitable for conducting the assay. The culture medium or body fluid containing the reporter gene product (analyte) is added to the microtitre plate to allow binding of the analyte. Addition of the same antibody or antiserum that has been conjugated to an enzyme, commonly horseradish peroxidase, is used as a second antibody. Addition of a suitable substrate, preferably one producing a colour product following conversion by the enzyme is used to quantify the analyte in proportion to how much second antibody conjugate has been bound.

(ii) Competitive ELISA. In an alternative form the tissue culture medium or the body fluid (analyte) sample containing the tagged lipocalin is bound to a support suitable for conducting the assay. In a separate reaction a limited standard amount of antibody specifically recognising the reporter gene product is added to a separate aliquot of the same and allowed to bind. This is added to the analyte bound to the support to allow remaining free antibody to bind. A second, enzyme conjugated antibody against for example the Fc region of the first antibody is allowed to bind and the colorimetric

readout can be used to quantify the analyte whereby the degree of colour change is inversely proportional to the level of analyte in the sample.

(iii) Western blot analysis

Transfected cell homogenates were prepared by incubation of cells in homogenization buffer (140mM NaCl, 50mM Tris-HCl pH7.5, 1mM EDTA, 1% Triton-100) for 30 minutes on ice. Following a brief centrifugation to remove insoluble material the cleared supernatants were assayed for protein content. A volume equivalent to 40µg cell extract and an equal volume of cell medium were subjected to SDS-PAGE and blotted onto nitrocellulose (Schleicher and Schuell, Dassel, Germany) membrane using a semi-dry blotting apparatus (Bio-Rad, Richmond, CA). The membranes were blocked for 1 hour in blocking buffer (5% NFDM w/v in PBS) then incubated with myc mAb (Invitrogen Life Technologies, Carlsbad, CA) diluted in blocking buffer for 2 hours with continuos agitation. After a series of washes in PBST (PBS plus 0.05% Tween-20), the membrane was incubated in an anti-mouse antibody conjugated to HRP diluted in blocking buffer for one hour with agitation, and after another series of washes in PBST the HRP activity was developed using an ECL kit (Pierce, Rockford, IL) and captured on autoradiographic film (Kodak).

(iv) Fluorescence polarisation. The antibody specifically recognising the reporter lipocalin protein is conjugated with fluorescein and mixed with the analyte produced. This method quantifies the analyte by direct measurement of the amount of antibody-antigen complex present. This method may also be adapted to measure any protein-protein interaction.

2. Release of a labelled substrate. E.g. radioactive (CAT) or fluorometric, colormetric.

Detection of conversion of substrate due to enzymatic activity of the lipocalin reporter protein produced. The nature of substrate conversion may or may not fall into one or more of the following event categories: Proteolysis, phosphorylation, acetylation, sulphation, methylation

3. Detection of multiple substrates. Where a multiple of lipocalin reporter proteins are used methods suitable for detection of such events could include but not necessarily be limited to:

(i) Mass spectrometry

(ii) Nuclear magnetic resonance (NMR)

In a preferred embodiment of the invention there is provided a method of detecting a reporter gene activation event, comprising the steps of:

1. Transfecting a cell or microinjecting the pronucleus of a fertilised mouse egg with a nucleic acid sequence encoding a lipocalin protein tagged with a peptide or protein as described above in accordance with the first, second, third, or fourth aspects of the invention. Optionally use the microinjected egg or transfected mouse ES cell line;

2. Exposing the transfected cell, cell line or transgenic non human animal to a stimulus which may or may not cause a change in metabolic status resulting alteration in gene expression; and.

3. Using a suitable assay to determine the level expression of the tagged lipocalin reporter, for example using detection methods such as ELISA, RIA, Mass spectrometry, NMR, telemetric methods.

In step (1), the detectable lipocalin protein may be a heterologous protein to the cell in which the nucleic acid construct is expressed. Such an "untagged" lipocalin reporter protein may not therefore need a peptide or protein tag for detection.

Methods and uses in accordance with the present invention offer significant advances in investigating any area in which modified gene expression plays a significant role. Such peptide tagged lipocalin genes will be of use in cells and transgenic animals to detect activity of selected genes. Specific applications include but are not restricted to:

1. Providing a rapid and robust in vivo screening system for assessing the potential toxic effects of chemicals.
2. Provide information on the mechanism of toxicity. Such information could be used to eliminate compounds from a selection process or suggest possible modifications to a compound.
3. Provide information on the effect of combinations of compounds.
4. Allow monitoring of variation in reporter gene expression over time by measuring levels of reporter(s) in urine at different time intervals.
5. Assessment of changes in gene expression associated with pathogenic infection.
6. Assessment of changes in gene expression associated with neurological, cardiovascular and metabolic diseases.
7. Assessment of changes in gene expression associated with cancer.
8. Provide information allowing validation of drug target selection e.g. by matching reporter expression profile to actions of toxins whose mechanism is defined and understood.
9. Use for evaluating compounds as therapeutic strategies aimed at reversing a toxic, metabolic, or degenerative phenotype.
10. Assessment of changes in gene expression resulting from environmental and/or behavioural changes.

Preferred features for the second and subsequent aspects of the invention are as for the first aspect *mutatis mutandis*.

The present invention will now be described with reference to the following examples which are present for the purposes of illustration only and should no be construed as

being limited with respect to the invention. Reference in the application is also made to a number of drawings in which:

FIGURE 1 shows the position of the peptide tag at the amino terminal or carboxy terminal or inserted internally with respect to the amino acid sequence of the lipocalin reporter protein

FIGURE 2 shows the plasmid map for pα1ATBLG

FIGURE 3 shows the plasmid map for pXC3'MycMUP

FIGURE 4 shows the plasmid map for pcDNA.3'mycMUP

FIGURE 5 shows the plasmid map for pX4T.3'MYCMUP

FIGURE 6 shows the results of expression of Myc tagged MUP

Example 1: Preparation of pα1ATBLG

The α1AT promoter (350bp) was excised from a1AT/CAT (Yull *et al Transgenic Res.* 4 70-74 (1995)) as a HindIII SmaI fragment and inserted into pBlueα1AT. Digestion of this with EcoRV and XhoI allowed direct insertion of the α1AT promoter into pXen6.S (Simon Temperley, CXR Biosciences) digested with the same enzymes. The microinjection fragment was purified after digestion of the plasmid with pα1ATBLG (shown in Figure 2).

Example 2: Prepraration of pX4T3'MycMUP

A XhoI/KpnI fragment encoding amino terminal c-Myc tagged mouse MUP was inserted into pXAM4 (CXR Biosciences) effectively placing it under the control of the CMV promoter. pXAM4 was previously constructed by inserting a PCR generated fragment containing the CMV promoter as a BamH1-XhoI fragment into a pSP72 (Promega) multiple cloning site which had been modified by addition of a linker

which added restriction sites allowing insertion of additional fragments downstream of the CMV promoter sequence.

Example 3: Preparation of pXC3'MycMUP

A 2.5kb DNA fragment encompassing the murine Cyp1A1 promoter and upstream sequences was inserted into SstII/XhoI digested pX4T.3'MycMUP (Thomas McCartney, CXR Biosciences) to engineer a reporter vector capable of expressing COOH terminally c-Myc tagged MUP upon induction of the CYP1A1 promoter using a suitable inducing agent, if the construct is used to transfect a suitable cell line or to generate a transgenic animal.

Example 4: pcDNA.3'MycMUP

A DNA fragment encompassing the COOH terminally c-Myc tagged MUP was excised from pX4T.3'Myc (Thomas McCartney, CXR Biosciences) to engineer an expression vector capable of constitutive expression of c-Myc tagged MUP if used to transfect a suitable cell line or to generate a transgenic animal.

Example 5: Expression of Myc-MUP

Constructs were tested by transient transfection of a 90% confluent monolayer of Hepa1-6 cells in a T-25 flask using 6ug of DNA in accordance with the protocol supplied with Lipofectamine transfection reagent (Invitrogen).

Cells and 5ml of medium were harvested 48 hours post-transfection. Total protein from the cell pellets was obtained using 1ml TRI reagent (Sigma) per pellet in accordance with directions. Cellular protein was further purified using the PlusOne SDS-PAGE Clean-Up Kit (Amersham) in accordance with directions. Correspondingly, protein was purifed from 100µl samples of growth medium from each transfected cell batch using the PlusOne SDS-PAGE Clean-Up Kit in accordance with directions.

Cell extracts and culture medium from Hepa1 cells transfected with constructs designed to constitutively express NH3 and COOH terminally Myc tagged MUP

coding sequences from the CMV promoter (2nd and 3rd lanes from left respectively in both left and right panels; plasmids X4T5'MycMUP and X4T3'MycMUP respectively) were subject to SDS-PAGE. Results shown in Figure 6

Western blot analysis by probing with antibody against c-Myc showed the presence of COOH terminally tagged MUP in both cell extract and medium of Hepa1 cells (3rd lane from left in both left and right hand panels). Results shown in Figure 6

25% of the total cellular protein samples and the entire protein sample derived from the growth medium were analysed by SDS-PAGE followed by western blot in accordance with equipment manufacturer's (BIO-RAD) directions. The blot was probed using the murine monoclonal Anti-Myc antibody 9E10 (Sigma) in conjunction with anti-mouse Ig HRP conjugated antibody (Amersham). Visualisation was performed using ECL reagent (Amersham) in accordance with directions.

CLAIMS

1. A nucleic acid construct comprising (i) a nucleic acid sequence encoding a member of the lipocalin protein family, and (ii) and nucleic acid sequence encoding a peptide sequence of from 5 to 250 amino acid residues

2. A nucleic acid construct as claimed in claim 1, in which the lipocalin is selected from the group consisting of: ovine betalactoglobulin (BLG) (accession No. X12817), murine major urinary protein (MUP) (accession No. NM 031188) and rat α-2-urinary globulin (α-2u) (accession number M27434).

3. A nucleic acid construct as claimed in claim 1 or claim 2, in which peptide sequence is an epitope,

4. A nucleic acid construct as claimed in claim 3, in which the epitope is from the c-myc protein or the simian virus V5 protein.

5. A nucleic acid construct as claimed in any one of claims 1 to 4, in which the construct additionally comprises a promoter element upstream of the (i) a nucleic acid sequence encoding a member of the lipocalin protein family, and (ii) and nucleic acid sequence encoding a peptide sequence of from 5 to 250 amino acid residues.

6. A nucleic acid construct as claimed in claim 5, in which the promoter element may be selected from one of the following groups consisting of :

(i) c-myc, p21/WAF-1, MDM2, Gadd45, FasL, GAHSP40, TRAIL-R2/DR5, BTG2/PC3;

(ii) MnSOD, CuZnSOD, IκB, ATF4, xanthine oxidase, COX2, iNOS, Ets-2, FasL/CD95L, γGCS, ORP150.

(iii) Lrg-21, SOCS-2, SOCS-3, PAI-1, GBP28/adiponectin, α-1 acid glycoprotein, metallothioneine I, metallothioneine II, ATF3, IGFbp-3, VDGF and HIF1α.

(iv) Gadd 34, GAHSP40, TRAIL-R2/DR5, c-fos, CHOP/Gadd153, APAF-1, Gadd45, BTG2/PC3, Peg3/Pwl, Siah1a, S29 ribosomal protein, FasL/CD95L, tissue tranglutaminase, GRP78, Nur77/NGFI-B, CyclophilinD, p73 and Bak.

(v) a promoter from a xenobiotic metabolising cytochrome p450 enzymes from the 2A, 2B, 2C, 2D, 2E, 2S, 3A, 4A and 4B gene families.

(vi) a synthetic promoter sequence comprised of a minimal eukaryote consensus promoter operatively linked to one or more response elements selected from the group consisting of the aryl hydrocarbon (Ah)/Ah nuclear translocator (ARNT) receptor response element, the antioxidant response element (ARE), the xenobiotic response element (XRE).

7. A nucleic acid construct comprising a stress inducible promoter operatively isolated from a nucleic acid sequence encoding a member of the lipocalin protein family by a nucleotide sequence flanked by nucleic acid sequences recognised by a site specific recombinase, or by insertion such that it is inverted with respect to the transcription unit encoding a member of the lipocalin protein family, in which the construct addtionally comprises a nucleic acid sequence comprising a tissue specific promoter operatively linked to a gene encoding the coding sequence for the site specific recombinase.

8. A nucleic acid construct as claimed in claim 7, in which the site specific recombinase sequences are two *lox*P sites of bacteriophage P1.

9. A host cell transfected with a nucleic acid construct according to any one of claims 1 to 8.

10. A transgenic non-human animal in which the cells of the non-human animal express the protein encoded by the nucleic acid construct according to any one of claims 1 to 8.

11. A transgenic non-human animal as claimed in claim 10, in which the non-human animal is a mammal

12. A transgenic non-human mammal as claimed in claim 11, in which the mammal is a mouse

13. The use of a nucleic acid construct according to any one of claims 1 to 8 for the detection of a gene activation event resulting from a change in altered metabolic status in a cell *in vitro* or *in vivo*.

14. A use as claimed in claim 13, in which the gene activation event is the induction of toxicological stress, metabolic changes, or viral, bacterial, fungal or parasitic infection.

15. The use of a nucleic acid construct comprising a nucleic acid sequence encoding a member of the lipocalin protein family, wherein said lipocalin protein is heterologous to the cell in which it is expressed, for the detection of a gene activation event resulting from a change in altered metabolic status in a cell *in vitro* or *in vivo*.

16. A use as claimed in claim 15, in which the gene activation event is induction of toxicological stress, metabolic changes, or viral, bacterial, fungal or parasitic infection.

17. A method of detecting a gene activation event in a cell *in vitro* or *in vivo*, comprising assaying a host cell stably transfected with a nucleic acid construct in accordance with any one of claims 1 to 8, or a transgenic non-human animal according to any one of claims 10 to 12, in which the cell or animal is subjected to a gene

activation event that is signalled by expression of a peptide tagged lipocalin reporter gene.

18. A method of detecting a gene activation event in a cell *in vitro* or *in vivo*, comprising assaying a host cell stably transfected with a nucleic acid construct comprising a nucleic acid sequence encoding a member of the lipocalin protein family, wherein said lipocalin protein is heterologous to the cell in which it is expressed, or a transgenic non-human animal whose cells express such a construct, in which the cell or animal is subjected to a gene activation event that is signalled by expression of a peptide tagged lipocalin reporter gene.

19. A method of screening for, or monitoring of toxicologically induced stress in a cell or a cell line or a non-human animal, comprising the use of a cell, cell line or non human animal which has been transfected with or carries a nucleic acid construct according to any one of claims 1 to 8.

20. A method for screening and characterising viral, bacterial, fungal, and parasitic infection comprising the use of a cell, cell line or non human animal which has been transfected with or carries a nucleic acid construct according to any one of claims 1 to 8.

21. A method for screening for cancer, inflammatory disease, cardiovascular disease, metabolic disease, neurological disease and disease with a genetic basis comprising the use of a cell, cell line or non human animal which has been transfected with or carries a nucleic acid construct according to any one of claims 1 to 8.

Figure 1. Tagged lipocalin reporter expression vector

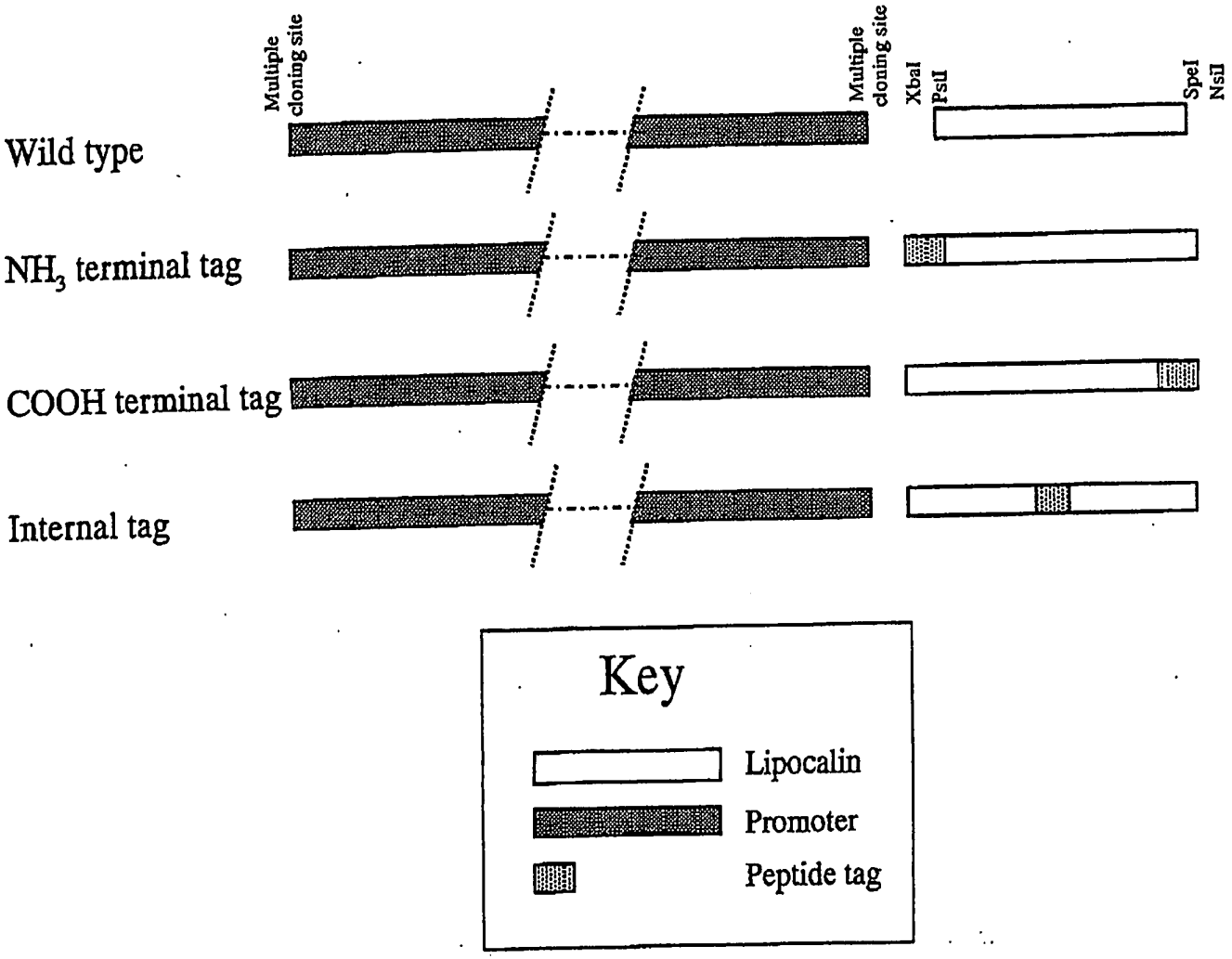
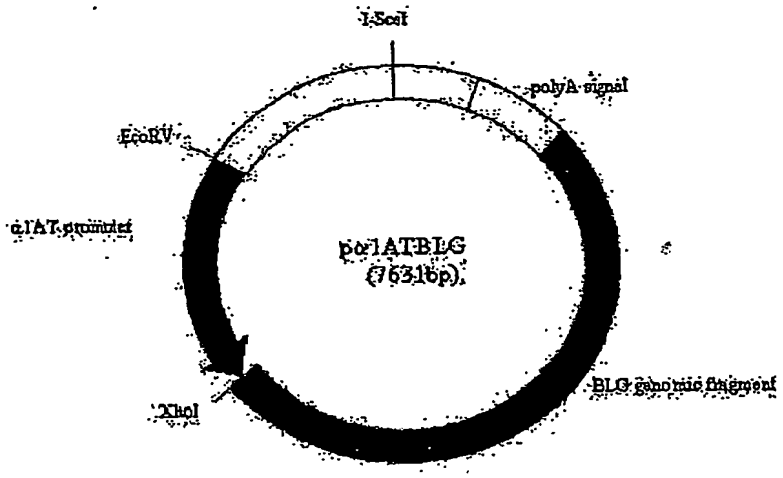


Figure 1



The α1AT promoter (350bp) was excised from a1AT/CAT (Yull et al., 1995) as a HindIII SmaI fragment and inserted into pBluescript digested with HindIII and HincII to yield plasmid pBlueα1AT. Digestion of this with EcoRV and XhoI allowed direct insertion of α1AT promoter into pXen6.S (Simon Temperley, CXR) digested with the same enzymes. The microinjection fragment was purified after digestion of the plasmid with pα1ATBLG.

References:
Yull, F.E., Wallace, R.M., Clark, A.J. (1995) *Transgenic Research* 4, 70-74.

Figure 2

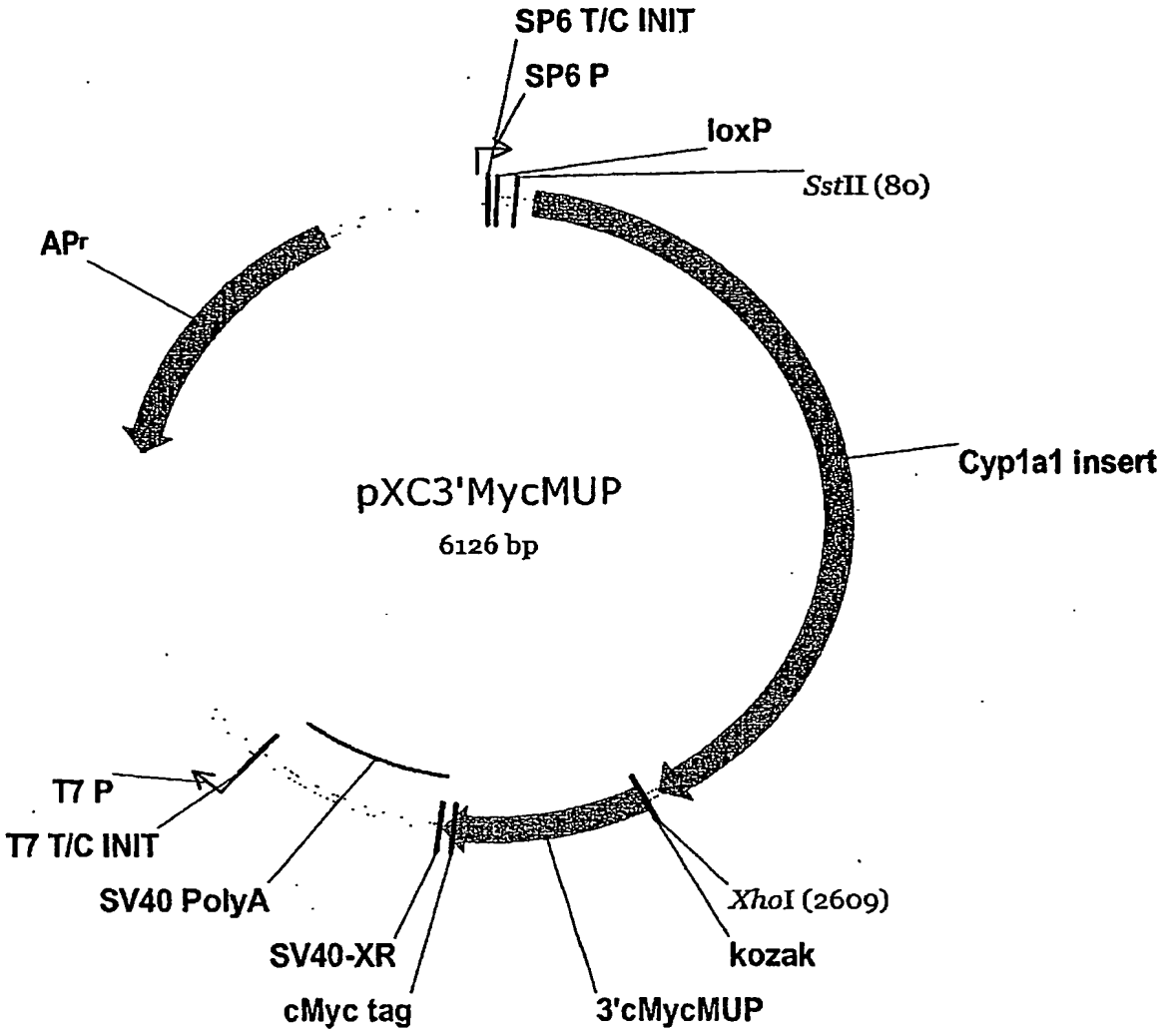


Figure 3

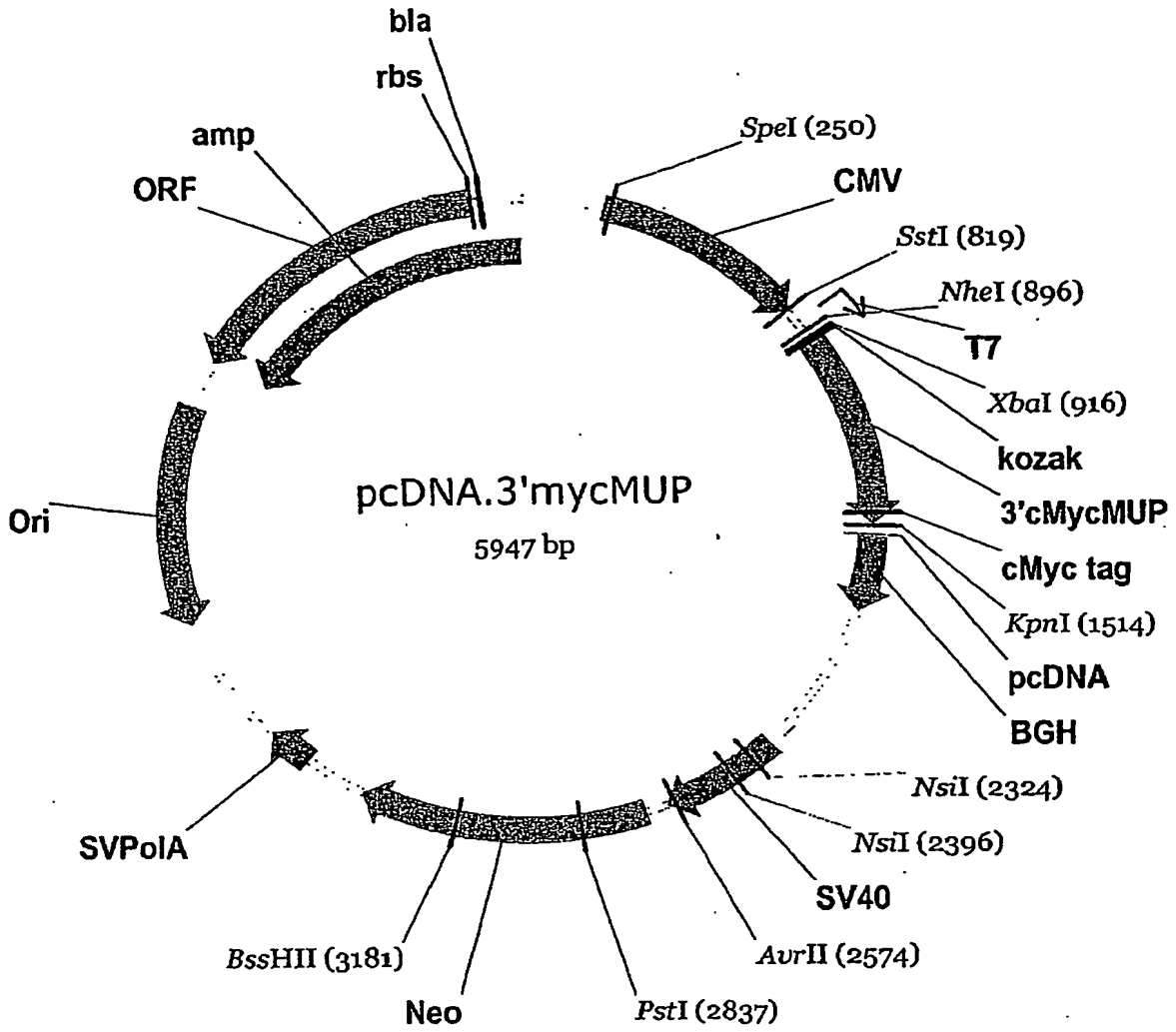
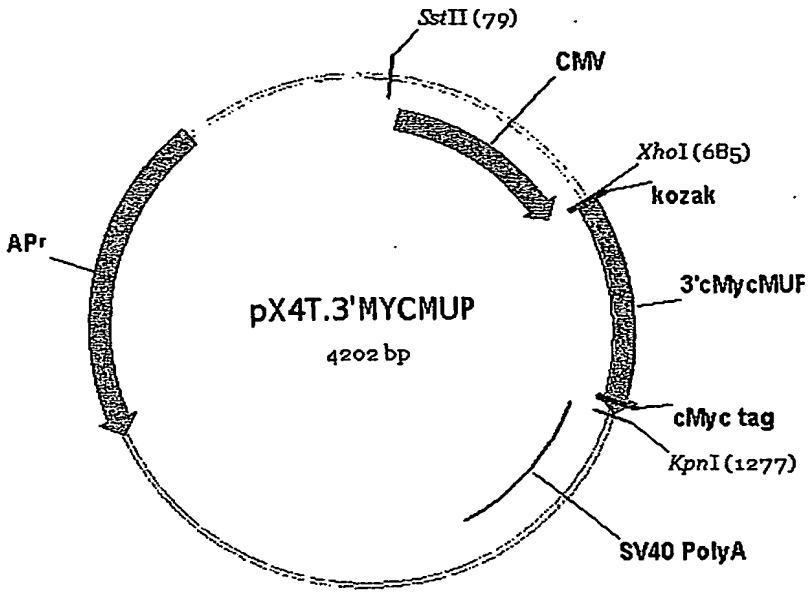


Figure 4



Generation of pX4T.3'MYCMUP: A XhoI/KpnI fragment encoding amino terminal c-Myc tagged mouse MUP was inserted into pXAM4 (CXR Biosciences) effectively placing it under the control of the CMV promoter. pXAM4 was previously constructed by inserting a PCR generated fragment containing the CMV promoter as a BamH1-XhoI fragment into a pSP72 (Promega) multiple cloning site which had been modified by addition of a linker which added restriction sites allowing insertion of additional fragments downstream of the CMV promoter sequence.

Figure 5

Myc Tagged MUP

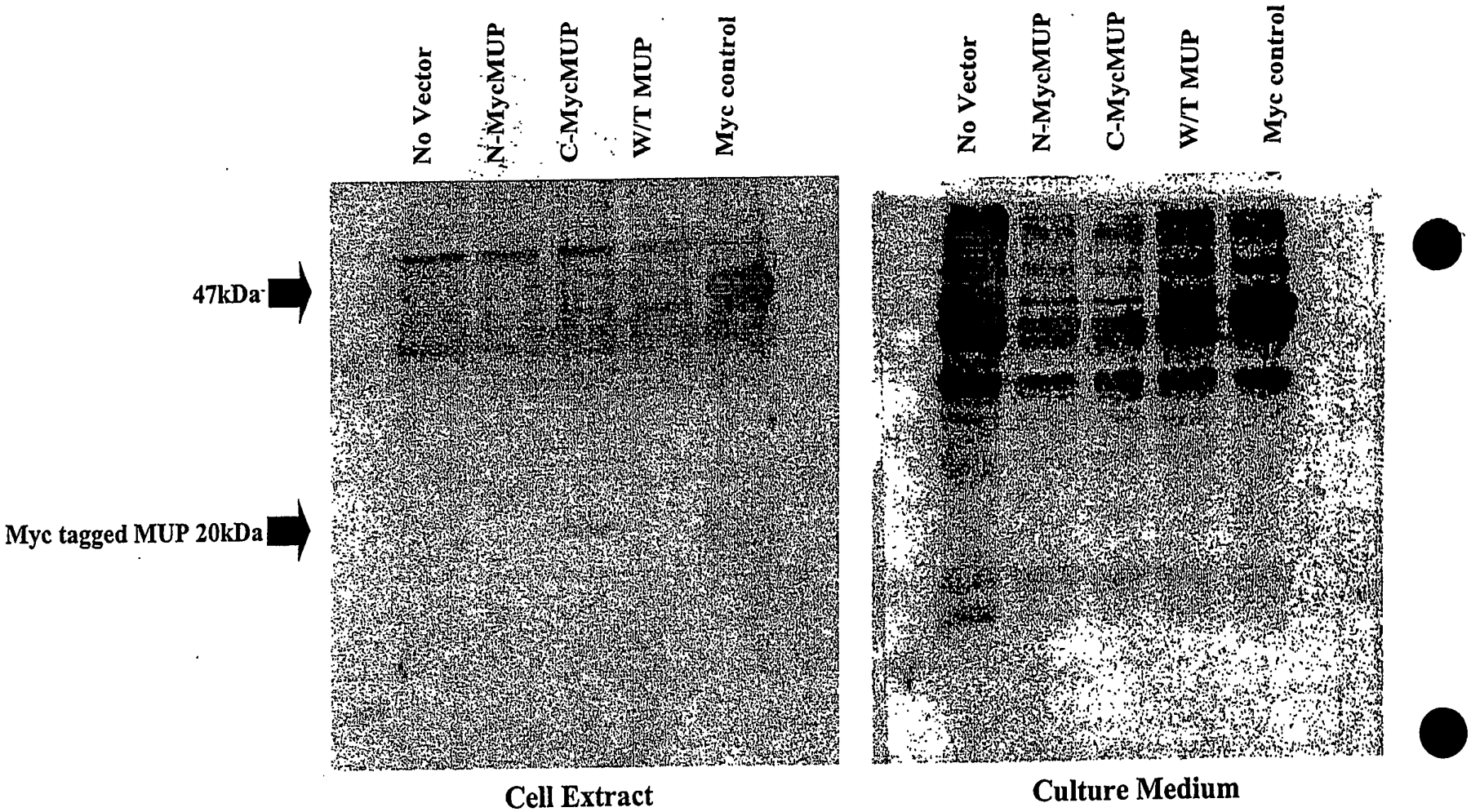


Figure 6

Cell extracts and culture medium from Hepa1 cells transfected with constructs designed to constitutively express NH3 and COOH terminally Myc tagged MUP coding sequences from the CMV promoter (2nd and 3rd lanes from left respectively in both left and right panels; plasmids X4T5'MycMUP and X4T3'MycMUP respectively) were subject to SDS-PAGE.
Western blot analysis by probing with antibody against c- Myc showed the presence of COOH terminally tagged MUP in both cell extract and medium of Hepa1 cells (3rd lane from left in both left and right hand panels).